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ORIGINAL ARTICLE

Characterization of Shiga toxin-producing *Escherichia coli* isolated from dairy cows in Argentina

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Abstract

Aims: To feno-genotypically characterize the Shiga toxin-producing *Escherichia coli* (STEC) population in Argentinean dairy cows.
Methods and Results: From 540 STEC positive samples, 170 isolates were analyzed by multiplex PCR and serotyping. Of these, 11% carried *stx1*, 52% *stx2* and 37% *stx1/stx2*. The *ehxA*, *saa* and *eae* were detected in 77%, 66% and 3%, respectively. Thirty-five per cent of strains harboured the profile *stx1, stx2, saa, ehxA* and 29% *stx2, saa, ehxA*. One hundred and fifty-six strains were associated with 29 different O serogroups, and 19 H antigens were distributed among 157 strains. STEC O113:H21, O130:H11 and O178:H19 were the most frequently found serotypes. The STEC O157:H7 were detected in low rate and corresponded to the $stx2^+$, eae^+, $ehxA^+$ virulence pattern.
Conclusions: We detected a diversity of STEC strains in dairy cattle from Argentina, most of them carrying genes linked to human disease.
Significance and Impact of the study: The non-O157 STEC serotypes described in this study are associated worldwide with disease in humans and represent a risk for the public health. For this, any microbiological control in dairy farms should be targeted not only to the search of O157:H7 serotype.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is an important foodborne pathogen associated with both outbreaks and sporadic cases of human disease, ranging from uncomplicated diarrhoea to haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). Typically, STEC affects children, elderly and immuno-compromised patients (Pearce *et al.* 2004). In Argentina, HUS is endemic, with 400 new cases per year and an incidence of 13·9/100 000 children under 5 years old (Ministry of Health 2006).

The term STEC refers to *E. coli* serotypes capable of producing Shiga toxin type 1 (Stx1), type 2 (Stx2) or both, encoded by *stx1* and *stx2* genes, respectively. These strains are likely to produce putative accessory virulence factors such as intimin (encoded by *eae*), an enterohaemolysin (EhxA) and an autoagglutinating protein commonly associated with *eae*-negative strains (Saa), both encoded by an enterohaemorrhagic plasmid (Paton *et al.* 2001).

STEC has been found in cattle (Beutin *et al.* 2004; Padola *et al.* 2004; Aidar-Ugrinovich *et al.* 2007), and several studies in Argentina have confirmed that cattle are the principal reservoir of non-O157:H7 serotypes (Sanz *et al.* 1998; Parma *et al.* 2000; Meichtri *et al.* 2004; Padola *et al.* 2004; Fernández *et al.* 2009); many of them have been involved in HUS and HC outbreaks in other countries (Bettelheim 2007).

In Argentina, studies in children with HUS identified O157:H7 serotype as the most prevalent, although others found this serotype with low prevalence (López *et al.* 1998; Rivas *et al.* 2006). However, as STEC non-O157 strains are more prevalent in animals and as contaminants in foods, humans are probably more exposed to these strains (Beutin *et al.* 2004; Blanco *et al.* 2004). Moreover, infections with some non-O157 STEC serotypes, such as O26:H11 or H-, O91:H21 or H-, O103:H2, O111:H-,

O113:H21, O118:H16, O121:H19, O128:H2 or H-, O130:H11, O141:H19, O145:H28 or H-, O146:H21, O163:H19, O172:NM and O178:H19, are frequently associated with severe illness in humans (Beutin *et al.* 2004; Blanco *et al.* 2004; Bettelheim 2007). Moreover, non-O157 STEC includes hundreds of serotypes associated with human infections (http://www.microbionet.com.au/vtectable.htm). This reinforces the idea of using detection methods that do not exert selection pressure for any particular serotype.

Transmission of STEC to humans occurs through the consumption of undercooked meat, vegetables and water contaminated by faeces of carriers and by person-to-person contact. Dairy farms may contribute to the risk of human STEC infection in many ways, such as the consumption of milk, whether pasteurized or not. Binderova and Rysanek (1999) have detected *E. coli* O157:H7 after high-temperature, short-time (HSTS) pasteurization, showing that the pasteurization or faulty pasteurization may not destroy all the foodborne pathogens in milk (Gunasekera *et al.* 2002) and does not inactivate Stx2 (Rasooly and Do 2010). Zweifel *et al.* (2010) detected a notable proportion of non-O157 STEC serotypes associated with human infections in semi-hard and hard raw milk cheese.

Taking into account these facts and the lack of data on the occurrence of STEC in dairy cattle from Argentina, this study aimed to isolate and characterize the STEC serotypes from cows in five dairy farms in Argentina.

Materials and methods

Samples, bacteriological procedures and virulence genes analysis

The 1440 samples were obtained by rectal swab from dairy cows belonging to five farms (named A, B, C, D and E). The cows were sampled at random in each dairy farm in different seasons of year, but the sampled animals were not the same in each season because the dairy cows were six months in milking before the dry period.

The rectal swabs of the 1440 samples were plated directly in MacConkey agar plates by incubating at 37°C for 24 h. An aliquot of confluent growth was inoculated into 30 ml of Luria–Bertani broth, incubated with shaking at 37°C for 4 h and processed for DNA extraction (Padola *et al.* 2004). Multiplex PCR was used to detect *stx1* and *stx2* genes (Paton and Paton 2002; Fernández *et al.* 2009). Primer sequences and experimental conditions for *stx1* and *stx2* amplification were indicated by Paton and Paton (2002). Five hundred and forty samples showed positive PCR results for one or both *stx1* and *stx2* and were considered STEC-positive (Fernández *et al.* 2009). They were then processed to isolate them and for feno-genotypical characterization.

For isolation of *E. coli* O157:H7, STEC positive samples were tested for the presence of the *eae-γ1* gene and then subjected to O157-specific immunomagnetic separation (IMS) kit (Dynal, Oslo, Norway) following the manufacturer's instructions. The concentrated samples were inoculated onto sorbitol-MacConkey plates, supplemented with 2·5 mg l^{-1} potassium tellurite and 0·05 mg l^{-1} cefixime (CT-SMAC). All negative colonies for sorbitol fermentation were confirmed as *E. coli* O157 with O157 latex (Oxoid Ltd.) particles (Padola *et al.* 2004).

For isolation of STEC non-O157, *stx* positive and *eae-γ1* negative samples were cultured on MacConkey agar plates. Ten to 200 separate colonies per sample were analysed by multiplex PCR for the presence of *stx1, stx2*, *eae*, *saa* and *ehxA* (Padola *et al.* 2004)

Amplification products were analysed by submarine gel electrophoresis (1·5% agarosa) and UV transillumination. Experimental conditions for *stx1*, *stx2*, *eae*, *eae-γ*, *saa* and *ehxA* amplification were indicated in previous papers (Woodward *et al.* 1992; Parma *et al.* 1996; Padola *et al.* 2004).

Serotyping

Screening for O-antigens was performed by microagglutination technique as described by Guinée *et al.* (1981) and modified by Blanco *et al.* (1992, 1996) with a kit of 70 antisera received from the Laboratorio de Referencia de *E. coli* (LREC) (Lugo, Spain). H antigens were determined by tube agglutination technique with 56 antisera (Statens Serum Institute, Copenhagen, Denmark) (Orskov and Orskov 1984). The nontypeable strains (ONT:HNT) were serotyped at Instituto Adolfo Lutz, Sao Paulo, Brazil, by tube agglutination test (Ewing 1986) using O (O1–O181) and H (H1–H56) antisera.

All STEC strains were processed for O serogroup determination, while H serotyping was performed only on those strains that, having been isolated from the same sample, differed in either one virulence factor or the O serogroup.

Results

Genetic profiles

In this study, from 540 STEC positive samples, we could isolate and characterize 170 strains. Multiplex PCR showed that 19/170 (11%) of the isolates carried *stx1* genes, 89/170 (52%) possessed *stx2* and 62/170 (37%) carried both *stx1/stx2*. Concerning the other virulence

factors, *ehxA*, *saa* and *eae* genes were detected in 131/170 (77%), 112/170 (66%) and 5/170 (3%) of the isolates, respectively. None of 112 *saa*-positive strains carried the *eae* genes, and 87% of *ehxA*-positive isolates were *saa*-positive.

Sixty (35%) isolates harboured *stx1, stx2, saa, ehxA,* 50 (29%) carried *stx2, saa, ehxA* and 32 (19%) harboured only *stx2*. Specifically, the farms A, C and D showed a high rate for the virulence profiles *stx1, stx2, saa* and *ehxA*. In the farm B, the main virulence profile was *stx2, saa, ehxA*, while in the farm E it was *stx2. A*ll the dairy farms showed high rates for the profile *stx2, saa, ehxA* (Table 1).

STEC serotypes

Among the 170 STEC isolates, 156 strains were associated with 29 different O serogroups (O2, O3, O5, O8, O11, O22, O26, O37, O39, O46, O64, O74, O79, O84, O88, O91, O105, O113, O130, O136, O139, O141, O157, O163, O166, O168; O171, O178, O179) and 14 were considered O nontypeable (NT). Nineteen H antigens (H2, H6, H7, H8, H10, H11, H16, H18, H19, H20, H21, H25, H27, H28, H38, H39, H41, H46, H49) were distributed among 157 strains, while 12 isolates were nonmotile (H-) and 1 H?. With the exception of O74:H28, O74:H39, O157:H7, O141:H8, O171:H2 and ONT:H19 serotypes, which lacked the ability to ferment sorbitol, all remaining STEC strains were sorbitol fermenters. The serophatotypes (association between virulences genes and serotypes) are shown in Table 2.

STEC O113:H21, O130:H11 and O178:H19 were the most frequently identified serotypes in all farms and were detected with higher frequency during warm seasons than in cold seasons. STEC O37:H10, O136:H- and O166:H25 occurred only in particular farms. The STEC O157:H7 were detected in low rate in dairy farms A and B and corresponded to the $stx2^+$, eae^+, $ehxA^+$ virulence pattern (Table 2).

Discussion

In this study, the *stx2* gene was the predominant *stx* type (52%), in agreement with previous studies in cattle and humans from Argentina (Parma *et al.* 2000; Meichtri *et al.* 2004; Padola *et al.* 2004; Rivas *et al.* 2006) and dairy cattle from other countries (Cobbold and Desmarchelier 2000; Irino *et al.* 2005; Fremaux *et al.* 2006). Stx2 is more cytotoxic than Stx1, and it has been demonstrated that Stx2 is associated with high virulence in humans (Fremaux *et al.* 2006; Rasooly and Do 2010).

A low proportion of STEC strains (3%) carried *eae* gene, in agreement with those obtained in grazing cows from Argentina by Sanz *et al.* (1998) (2%), in Australia by Cobbold and Desmarchelier (2000) (0·7%) or in Brazil by Irino *et al.* (2005) (1%), but differed from studies carried out in feedlot cattle by Padola *et al.* (2004) (38·6%) or in Spain and France (Blanco *et al.* 2004; Fremaux *et al.* 2006) and Brazil (Leomil *et al.* 2003) in which the percentage of *eae* genes were greater. Several researchers have underlined a strong association between carriage of the *eae* genotype and the capacity of STEC to cause severe human disease, especially HUS (Karmali 1989; Paton *et al.* 2001; Aidar-Ugrinovich *et al.* 2007). Nevertheless, intimin is not essential for pathogenesis, because outbreaks and a number of sporadic cases of HUS have been caused by *eae*-negative strains (Paton *et al.* 2001; Aidar-Ugrinovich *et al.* 2007). The *eae*-negative strains would be using other mechanism of adhesion to epithelial cells than those related to intimin. In fact, Paton *et al.* (2001) described a new virulence gene, *saa*, which may be an important virulence factor of *eae*-negative STEC strains. Jenkins *et al.* (2003) showed that there is no significant association between STEC isolated from patients with HUS and *saa*-positive strains; however, it has been reported that STEC O91:H21 and O113:H21 (*saa*-positive and *eae*-negative) are capable of colonizing the human gastrointestinal tract and cause HUS (Blanco *et al.* 2004; Aidar-Ugrinovich *et al.* 2007). In our present study, all

Table 1 Comparison of virulence profiles for STEC isolates from the different dairy farms

	Farm					
Virulence profile	A	B	C	D	E	Total
stx1	0/38 (0%)	1/20 (5%)	2/41 (5%)	0/50 (0%)	4/21 (19%)	7/170 (4%)
stx2	8/38 (21%)	3/20 (15%)	5/41 (12%)	7/50 (14%)	9/21 (43%)	32/170 (19%)
stx2, saa, ehxA	8/38 (21%)	11/20 (55%)	6/41 (14%)	19/50 (38%)	6/21 (28%)	50/170 (29%)
stx1, saa, ehxA	1/38 (3%)	0/20 (0%)	1/41 (2%)	0/50 (0%)	0/21 (0%)	2/170 (1%)
stx1, ehxA	3/38 (8%)	0/20 (0%)	6/41 (14%)	0/50 (0%)	0/21 (0%)	9/170 (5%)
stx2, ehxA	0/38 (0%)	0/20 (0%)	3/41 (7%)	0/50 (0%)	0/21 (0%)	3/170 (2%)
stx2, eae, ehxA	2/38 (5%)	1/20 (5%)	1/41 (2%)	1/50 (2%)	0/21 (0%)	5/170 (3%)
sxt1,stx2, ehxA	1/38 (3%)	0/20 (0%)	1/41 (2%)	0/50 (0%)	0/21 (0%)	2/170 (1%)
stx1, stx2, saa, ehxA	15/38 (39%)	4/20 (20%)	16/41 (38%)	23/50 (46%)	2/21 (9%)	60/170 (35%)

Table 2 Distribution of serotypes, according to farm and virulence profile of STEC isolates from dairy cattle

Farms	Serotypes	No. of isolates	Virulence markers
A	O8:H16	1	*stx1, stx2, saa, ehxA*
	O8:H20	1	*stx1, stx2, saa, ehxA*
	O46:H11	1	*stx1,stx2 ehxA*
	O46:H38	2	*sxt1, stx2, saa, ehxA*
	O64:H-	1	*stx1, ehxA*
	O79:H-	2	*stx1, stx2, saa, ehxA*
	O91:H21	1	*stx2, saa, ehxA*
	O113:H21	6	*stx2, saa, ehxA*
	O113:H21	1	*stx1, stx2, saa, ehxA*
	O130:H11	6	*stx1, stx2, saa, ehxA*
	O130:H11	1	*stx1, saa, ehxA*
	O136:H-	1	*stx1, ehxA*
	O141:H8	1	*stx1, ehxA*
	O157:H7	2	*stx2, eae, ehxA*
	O163:H19	1	*stx2, saa, ehxA*
	O178:H19	7	*stx2*
	ONT:H2	1	*stx1, stx2, saa, ehxA*
	ONT:H7	1	*stx2*
	ONT:H21	1	*stx1, stx2, saa, ehxA*
B	O?H7	1	*stx2*
	O22:H27	1	*stx2, saa, ehxA*
	O91:H21	1	*stx2, saa, ehxA*
	O105:H?	1	*stx1*
	O105:H18	1	*stx1, saa, ehxA*
	O113:H21	3	*stx2, saa, ehxA*
	O130:H11	3	*stx1, stx2, saa, ehxA*
	O157:H7	1	*stx2, eae, ehxA*
	O166:H25	1	*stx2, saa, ehxA*
	O178:H19	2	*stx2*
	O179:H-	1	*stx2, saa, ehxA*
	O179:H8	2	*stx2, saa, ehxA*
	ONT:H7	1	*stx1, stx2, saa, ehxA*
	ONT:H46	1	*stx2, saa, ehxA*
C	O3:H-	1	*stx1, ehxA*
	O5:H11	1	*stx2, eae, ehxA*
	O11:H-	1	*stx1, stx2, ehxA*
	O26:H21	1	*stx1, ehxA*
	O37:H10	1	*stx2, ehxA*
	O64:H-	2	*stx1, ehxA*
	O79:H38	1	*stx1, saa, ehxA*
	O84:H41	1	*stx2, ehxA*
	O88:H25	1	*stx1, stx2, saa, ehxA*
	O91:H21	3	*stx2, saa, ehxA*
	O105:H18	1	*stx1, stx2, saa, ehxA*
	O113:H21	3	*stx2, saa, ehxA*
	O130:H11	11	*stx1, stx2, saa, ehxA*
	O130:H11	1	*stx1, ehxA*
	O139:H2	1	*stx2*
	O141:H19	1	*stx1*
	O168:H-	1	*stx2, ehxA*
	O178:H19	4	*stx2*
	O178:H19	2	*stx1, stx2, saa, ehxA*
	ONT:H-	1	*stx1, stx2, saa, ehxA*
	ONT:H7	1	*stx1, ehxA*
	ONT:H19	1	*stx1*
D	O39:H49	1	*stx2,saa,ehxA*
	O74:H39	1	*stx2, eae, ehxA*
	O91:H21	10	*stx2, saa, ehxA*
	O105:H18	2	*stx1,stx2, saa, ehxA*
	O113:H21	6	*stx2, saa, ehxA*
	O130:H11	15	*stx1,stx2, saa, ehxA*
	O171:H2	2	*stx2*
	O178:H19	5	*stx2*
	O178:H19	4	*stx1,stx2, saa, ehxA*
	O178:H19	2	*stx2,saa,ehxA*
	ONT:H21	1	*stx1,stx2, saa, ehxA*
	ONT:H46	1	*stx1,stx2, saa, ehxA*
E	O2:H6	1	*stx1*
	O3:H-	1	*stx1*
	O74:H28	2	*stx2, saa, ehxA*
	O113:H21	1	*stx2, saa, ehxA*
	O130:H11	1	*stx1, stx2, saa, ehxA*
	O141:H19	2	*stx1*
	O178.H19	1	*stx1,stx2, saa, ehxA*
	O178:H19	9	*stx2*
	ONT:H11	1	*stx2, saa, ehxA*
	ONT:H21	1	*stx2, saa, ehxA*
	ONT:H46	1	*stx2, saa, ehxA*

the *saa*-positive strains (66%) were *eae*-negatives and mostly *ehxA*-positive. This study shows that 29% and 35% of STEC harboured the genotypic profile *stx2*, *saa*, *ehxA* and *stx1*, *stx2*, *saa*, *ehxA*, respectively. However, Padola *et al*. (2004) found a higher prevalence of strains carrying the virulence profile *stx*, *eae* and *ehxA* in feedlot cattle from Argentina.

Most surveys in dairy cattle have only focused on the detection of *E. coli* O157:H7. In this study, we have found a lower prevalence of O157:H7 (0·2%) than that in a previous study by Padola *et al.* (2004) in a feedlot from Argentina (6·8%) that shows higher prevalence of this serotype when the animals were sampled serially. In abattoirs from Argentina, Masana *et al.* (2010) found STEC O157 in 4·1 and 2·6% of faecal and carcasses samples, respectively. In these same abattoirs, the prevalence of non-O157 STEC in faecal and carcass samples was estimated as 22% and 9%, respectively. However, a low prevalence of O157:H7 was also found by LeJeune *et al.* (2006) who isolated this serotype in 0·6% of dairy cows from Ohio (USA). These data indicated that O157:H7 is an uncommon serotype for cattle in Argentina, although it has been isolated from cattle and human faeces by other authors in this country with the same virulence profile *stx2*, *eae*, *ehxA* (Orskov *et al.* 1987; Padola *et al.* 2004; Rivas *et al.* 2006). However, it is difficult to compare the prevalence of O157:H7 owing to the differences in methodology among laboratories.

In our study, several non-O157 serotypes have been isolated from animals in agreement with those isolated from Argentina and Brazil by Padola *et al.* (2004), Meichtri *et al.* (2004), Irino *et al.* (2005), Masana *et al.* (2010) and Timm *et al.* (2007). Furthermore, this study is the first, to our knowledge, to describe the *E. coli* serotypes O22:H27, O37:H10, O46:H11, O79:H38, O84:H41 and O139:H2, carrying *stx* genes (http://www.microbionet.com.au/vtectable.htm).

The O113:H21, O130:H11 and O178:H19 STEC were the serotypes most frequently identified, and they were widely distributed among the different dairy farms investigated in this study, with increasing prevalence in warm seasons.

The occurrence of the serotypes O8:H16, O91:H21, O113:H21, O171:H2, O178:H19, ONT:H- and ONT:H21 had not previously been found in dairy cattle in Argentina but had been isolated from beef cattle (Blanco *et al.* 2004; Meichtri *et al.* 2004; Padola *et al.* 2004) and meat (Blanco *et al.* 2004) in Argentina.

The serotypes O105:H18, O113:H21, O130:H11 and O178:H19 presents more than one genotypic profile, corroborating the high diversity of STEC identified by serotyping. Seropathotypes isolated in this study were O91:H21 *stx2, saa, ehxA*, O113:H21 *stx2, saa, ehxA*, O130:H11 *stx1 stx2, saa, ehxA*; *stx1, saa, ehxA* and *stx1, ehxA*, O163:H19 *stx2, saa, ehxA* and O178:H19 *stx2*; *stx2, saa, ehxA* had been isolated from human patients with HUS, diarrhoea or HC in several countries including Argentina (Blanco *et al.* 2004; Fremaux *et al.* 2006; Timm *et al.* 2007; Rivas *et al.* 2008).

The isolation and diversity of STEC serotypes found in this study have confirmed that Argentinean dairy cattle are an important reservoir of STEC. The serotypes carrying genes related to human diseases suggest a risk to the population. This should be taken into account in the control and prevention measures to minimize the risk of STEC foodborne infection in humans.

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